ORIGINAL ARTICLE

A Quantitative Assay for Lycopene That Utilizes Reduced Volumes of Organic Solvents[1]

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Lycopene is a potent antioxidant found in watermelon, tomato, and red grapefruit and may exert positive effects on human health. Spectrophotometric and HPLC techniques are commonly employed for analysis of lycopene content in food sources. A rapid and inexpensive spectrophotometric assay for lycopene is presented. This method requires 80% less organic solvents for release and extraction of lycopene from watermelon than do the existing procedures. Comparative analyses for 105 watermelons from 11 cultivars yielded results equivalent to those provided by larger-volume spectrophotometric assay procedures. Limited numbers of assays suggest that this reduced volume method may be applicable for tomatoes and tomato products. Published by Elsevier Science Ltd.

Key Words: carotenoids; lycopene; lycopene assay; phytonutrient.

INTRODUCTION

Lycopene is a vibrant red carotenoid that serves as an intermediate for the biosynthesis of other carotenoids and is found in moderate to high concentrations in such foods as tomato, watermelon, red grapefruit, and Brazilian guava (Stahl and Sies, 1996). Like its biosynthetic derivatives such as β-carotene, lycopene is an efficacious free radical scavenger (DiMascio *et al.*, 1989), and its presence in the diet positively correlates with reduced cancer incidence (Gerster, 1997; Giovannucci *et al.*, 1995; Giovannucci, 1999; Rao and Agarwal, 1998).

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As a food that is gastronomically pleasing and rich in lycopene, watermelon is a highly desirable source of this phytochemical. Previous research has determined that watermelon cultivars vary in lycopene content depending on genotype and environmental conditions (Perkins-Veazie *et al.*, 2001). Plant breeders, therefore, need an inexpensive but reliable method for determining lycopene content in individual watermelons. Conventional spectrophotometric assays employ a release of lycopene from tissue with polar organic solvents followed by extraction of the released lycopene into a nonpolar solvent such as hexane (Beerh and Siddappa, 1959; Adsule and Dan, 1979; Sadler *et al.*, 1990). Although reliable and simple, these conventional assays for lycopene require the use of sizeable quantities of volatile organic solvents for each sample. For routine use, this quickly multiplies into large volumes of hazardous waste that are costly and that must be disposed of under strict environmental guidelines.

HPLC, on the other hand, potentially affords separation/identification of individual carotenoids as well as their quantitation. Currently, however, HPLC is still subject to the vagaries of sample extraction/preparation, many sources of column- and solvent-generated artifacts, and a paucity of pure carotenoid standards (Kimura and Rodriguez-Amaya, 1999). Furthermore, HPLC is too expensive and too slow for the routine screening of fruit from the many plants produced in breeding programs designed to develop new cultivars.

In an effort to be both fiscally and environmentally responsible, we have scaled down and modified a conventional organic extraction assay in order to reduce significantly the quantities of organic solvents used. At the same time, the precision and accuracy of existing methods were maintained.

MATERIALS AND METHODS

Assay Reagents and Supplies

Reagent grade acetone and U.S.P. grade 95% ethanol were employed in the assays. Initially, HPLC-grade *n*-hexane (Fisher, Pittsburgh, PA) was used; later, this was substituted with a less expensive grade of Reagent Grade "hexanes" ($\sim 50\%$ *n*-hexane in 99.9% total hexane isomers) (Pharmaco, Brookfield, CT). No quantitative differences were observed in the lycopene content measured for the same melon puree when results from each of the two grades of hexane were compared.

Following the method of Sadler *et al.* (1990), Erlenmeyer flasks (250 mL) wrapped in aluminum foil and sealed with rubber stoppers were employed for the conventional assay procedure. For the reduced volume assay procedure, 40 mL amber screw-top vials (e.g., Fisher, Cat. No. 03-391-8F) were employed as extraction vessels. Vials were capped with reusable Teflon-lined lids. Amber glass negated the need to wrap each extraction vessel in foil since assays with and without the amber vials wrapped in foil yielded identical results. Tests of lycopene stability in the assay mixture held in the amber vials indicated that the absorbance of the lycopene in the hexane phase decreased at a rate of about 1% per hour if materials were held in the vials at room temperature under normal laboratory fluorescent lighting.

Plant Material and Sampling

Watermelons used for the study were from the 2001 harvest and were obtained from California, Texas, and Oklahoma. In addition, three watermelon cultivars were grown on research plots at Lane, OK. Six to 20 watermelons per cultivar were used

TABLE 1

Lycopene content (average $\pm$ S.D.) of red-fleshed watermelon cultivars

Cultivar	Number of melons sampled	Lycopene (mg/kg fresh wt.)
Seedless types		
HMX-8914	6	45.3 ± 6.1
Millenium	6	52.1 ± 3.3
Sugar Time	6	51.2 ± 7.0
Millionaire	6	52.1 ± 5.2
Tri-X-313	15	63.0 ± 5.5
Summer Sweet 5244	7	58.3 ± 4.1
Sugar Shack	20	52.2 ± 7.1
Hybrid seeded types		
Royal Sweet	6	39.1 ± 4.1
Sangria	7	58.5 ± 5.4
Summer Flavor 800	20	60.7 ± 7.1
Open-pollinated seeded type		
Allsweet	6	39.1 ± 3.8

from each of the 11 cultivars for a total of 105 individual watermelons (Table 1). Most cultivars were seedless triploids, but hybrid-seeded and open-pollinated seeded types were also included.

Prior to analysis, watermelon tissue was handled in one of the two ways. In one method, two 100 g tissue samples were removed from the center of the watermelon heart and stored at -80°C until assayed. Thirty gram samples of frozen watermelon tissue were ground with mortar and pestle and were then ground to a homogeneous puree with an electric tissue grinder (Brinkman Polytron Homogenizer, Westbury, NY). In the second method, 40 g samples were removed from the heart of a fresh watermelon and immediately ground to a homogeneous puree with an electric tissue grinder.

Fresh tomatoes were finely ground to a puree in an equal weight of distilled water with an electric tissue grinder before sampling and assay. Tomato products, particularly tomato paste and tomato catsup, were diluted three- to five-fold by weight with distilled water before sampling and assay in order to get assay responses in a reliable range.

Tissue purees were kept on ice and out of light after preparation and until assayed. Purees were stirred on ice on a magnetic stirring plate during replicate sampling. Sampling and weighing of replicates were performed in reduced room light.

Conventional Lycopene Assay

Total lycopene was measured on duplicate 2 g samples of each puree by the method of Sadler *et al.* (1990) modified as described by Perkins-Veazie *et al.* (2001). This assay requires 25 mL of 0.05% (w/v) butylated hydroxytoluene (BHT) in acetone, 25 mL of 95% ethanol, and 50 mL hexane per sample assayed.

Reduced Volume Lycopene Assay

To each amber vial were added: 5 mL of 0.05% (w/v) BHT in acetone, 5 mL of 95% ethanol, and 10.0 mL of hexane. The hexane was delivered with a volumetric pipette calibrated to deliver 10.0 mL at 20°C. Later assays employed volume dispensers to deliver the organic solvents (Dispensette^{TM} organic dispenser, BrandTech Scientific, Essex, CT). Both dispensing techniques yielded results comparable in precision and accuracy. Each puree was assayed in triplicate. A given volume of sample was

removed from the stirred puree and pipetted into its corresponding assay vial whose tare weight had been determined. Depending upon the balance utilized, the weight of sample was determined to the nearest 0.01 g or 0.001 g. The amount of sample used in the reduced volume assay can range from 0.4 to 0.6 g. At this range of sample weight, it is imperative to record the weight of sample added to the assay vial to (at least) the nearest 0.01 g in order to maintain an acceptable level of weighing precision. Vials were laid on their sides in a rectangular container, covered with a second container that contained ice and placed on an orbital shaker (Lab-Line Instrument Co., Melrose Park, IL) to mix at 180 rpm for 15 min. After 15 min of shaking, 3 mL of deionized water were added to each vial, and the samples shaken for another 5 min. Shaking was stopped, and vials were left at room temperature for 5 min to allow for phase separation. The absorbance of the hexane (upper) layer was measured in a 1 cm path length quartz cuvette at 503 nm versus a blank of hexane solvent. The lycopene content of tissue was then estimated by one of the relations:

$$\text{Lycopene (mol/kg tissue)} = \frac{A_{503}}{17.2 \times 10^4/\text{M} \times \text{cm}} \times \frac{1\text{L}}{10^3\,\text{mL}}$$
$$\times \frac{10.0\,\text{mL}}{\text{kg tissue}}$$
$$= \frac{A_{503} \times 5.81 \times 10^{-8}}{\text{kg tissue}},$$

$$\text{Lycopene (mg/kg tissue)} = \frac{A_{503}}{17.2 \times 10^4/\text{M} \times \text{cm}} \times \frac{536.9\,\text{g}}{\text{mole}}$$
$$\times \frac{1\text{L}}{10^3\,\text{mL}} \times \frac{10^3\text{mg}}{1\,\text{g}} \times \frac{10.0\,\text{mL}}{\text{kg tissue}}$$
$$= \frac{A_{503} \times 0.0312}{\text{kg tissue}} = \frac{A_{503} \times 31.2}{\text{g tissue}},$$

where the molar extinction coefficient of $17.2 \times 10^4/\text{M/cm}$ is that reported by Zechmeister *et al.* (1943) for lycopene in hexane. Most extinction coefficients that have been reported subsequently are within 1–2% of this value (e.g., DeRitter and Purcell, 1981). We chose to work with values of lycopene content expressed in terms of mg/kg since that (or the equivalent, µg/g) makes data handling easier and is a unit of concentration commonly used in the literature (Beerh and Siddappa, 1959; Perkins-Veazie *et al.*, 2001).

Although not the absorbance peak of greatest magnitude in hexane, the absorbance peak at 503 nm was used in order to minimize interference from other carotenoids. If generally accepted, nominal carotenoid contents of red-fleshed watermelon, fresh red tomato, and pink grapefruit are utilized (Holden *et al.*, 1999) together with molar extinction coefficients at 503 nm in hexane for those carotenoids (Zechmeister *et al.*, 1943; Zechmeister and Polgar, 1943), the potential error can be estimated if absorbance contributions by other carotenoids are ignored. Such a calculation suggests that constituent carotenoids other than lycopene will contribute to the absorbance at 503 nm <2% for red-fleshed watermelon, <4% for fresh red tomatoes, and <6% for pink grapefruit. These levels of possible lycopene overestimation are at or near the levels of uncertainty in the parameters used in the calculation and other parameters of the method. Thus, this or any extraction/ spectrophotometric assay for lycopene should provide reasonable results for those foods in which lycopene constitutes at least 70% of the constituent carotenoids.

The distribution and relative levels of carotenoids in a number of the watermelon samples were determined with HPLC and diode array detection by the method of Tonucci *et al.* (1995) to verify that the samples contained no unusual carotenoid distributions or aberrant levels of lycopene *cis*-isomers.

Statistical Analyses

Statistical analyses, linear regression analysis, analysis of variance, and mean and standard deviation determinations were performed with the aid of Statistica software, version 6 (StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

We first tested to see if the reduced volume method could fulfill two fundamental requirements. The first was that all of the lycopene present in a watermelon tissue puree could be extracted by the medium. The second was that the absorbance of the lycopene extracted into the hexane phase was a linear function of the amount of lycopene added to the assay system, i.e., did it obey Beer's law under this assay protocol? To test these two requirements, we prepared fresh watermelon tissue puree and added various quantities of the puree to vials containing the reduced volumes of extractants. Figure 1 illustrates the assay response as a function of the amount of sample (i.e., watermelon puree) extracted. The system appears to obey Beer's law to a maximum absorbance of ~ 1.8. Above this value, the assay response deviates sufficiently from linearity such that a sample of absorbance = 2.1 would underestimate the amount of lycopene by $\sim 6\%$. To test that the reduced volume protocol extracted all of the lycopene in the samples, the aqueous phase of each assay was re-extracted with a second 10 mL of hexane and the absorbance at 503 nm determined.
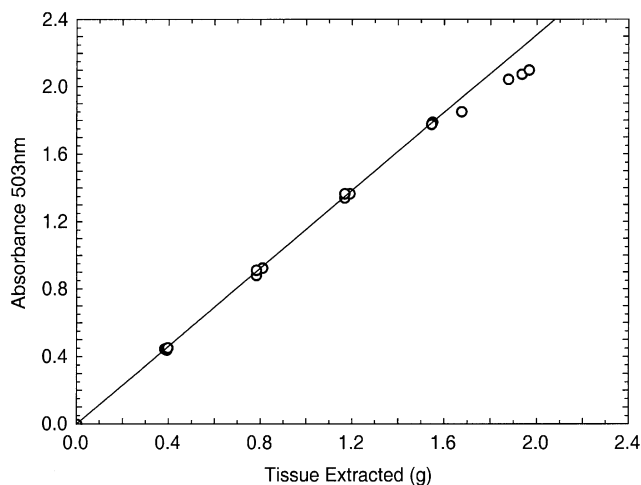

FIGURE 1. Absorbance response of the reduced volume assay to increasing levels of lycopene. The linear least-squares regression line was fitted to the data between 0 and 1.8 absorbance. The equation for this line is $y = 0.029 + 1.095x$. The regression coefficient r for this least squares linear fit is 0.998. The segment of the regression line between 0.5 and 1.5 absorbance represents the range of absorbance values expected from 0.5 g samples of watermelon tissue purees between 30 and 80 mg lycopene per kg fresh tissue. This range of lycopene is typical of red-fleshed watermelons.

The absorbances of the second hexane extracts ranged from <0.001 for the 0.4 g of tissue to <0.015 for 2.5 g of tissue (data not shown). These results are consistent with the requirement that all lycopene be released from the tissue and extracted into the hexane phase.

Practical validation of the reduced volume hexane extraction method was carried out by comparing the lycopene contents determined by this method for 105 individual watermelons with those determined by a conventional method. When lycopene values determined by the reduced volume method were plotted versus their corresponding values determined by conventional assay, a linear relation was obtained (Fig. 2(A)). This relation is consistent with the hypothesis that results from the two methods are directly comparable and supports the validity of the method. The percent difference in lycopene content as estimated by the two methods is also graphically presented (Fig. 2(B)). Percent difference is defined as the lycopene content determined by the reduced volume method minus that determined by the conventional method divided by the average of the two methods. The slope of a linear least-squares fit to the data was -0.012 with a correlation coefficient, r, of 0.057 which indicates that there was no linear relationship. No additional lycopene was detected in extracts of residues from samples that contained up to an equivalent of 200 mg/kg of lycopene in the initial extract. Thus, we believe that this slight negative slope can be considered to be zero and does not represent incomplete extraction of lycopene at higher levels. To test further for the absence of concentration-dependent error in lycopene estimation, means and variances of percent differences were calculated for the population of watermelons of lycopene content between 40 and 45 mg/kg and for the population of watermelons of 60–65 mg/kg lycopene. A comparison of these two means using analysis of variance ($P \leq 0.05$) indicated that there was no significant difference between the means of these two populations. This, too, is consistent with the premise that there is no concentration-dependent error in lycopene estimation by this reduced volume method. Under the assumption that the above premise is true, the average percent difference between the two methods for all determinations was –0.9% with a standard deviation of $\pm 2.4\%$ ($n = 105$). All watermelon cultivars demonstrated normal scatter of data about the mean percent difference. This suggests that no individual cultivar exhibited aberrant behavior in the assay. Also, the average amount of lycopene determined for each cultivar is similar to that previously reported (Holden *et al.*, 1999; Perkins-Veazie *et al.*, 2001).

The precision of the reduced volume assay procedure appears to be comparable to that of the conventional assay. For 228 watermelon samples assayed by the reduced volume method, the average standard error per triplicate was $1.22 \pm 0.84\%$ S.D.

Limited trials were conducted using the reduced volume assay with fresh tomatoes and tomato products such as tomato paste, tomato juice, and tomato catsup. In all instances, the reduced volume assay gave values statistically identical with those given by conventional assay (data not shown). No additional lycopene was extracted into the hexane layer upon re-extraction of the tomato residues. Although based on limited numbers of analyses, it appears that the reduced volume hexane extraction assay may be applicable to tomatoes and tomato products.

Like any quantitative assay procedure, there are several steps in the assay that demand careful attention in order to achieve the desired level of reliability. These include sample preparation and handling, sampling/weighing, and accurate dispensing of the requisite volume of hexane. It is imperative that the tissue be finely ground to a homogeneous puree and that it then be constantly stirred in order to facilitate reproducible sampling. Lycopene, even as part of a tissue puree, will
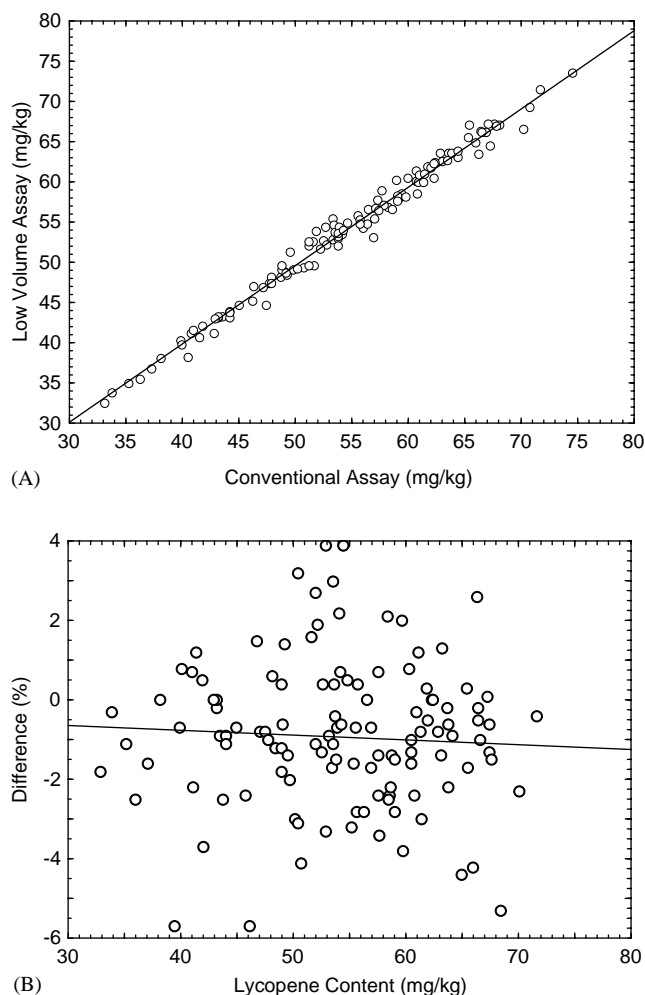
FIGURE 2. (A) Lycopene levels determined for 105 individual watermelons by the reduced volume assay compared with the corresponding levels determined by conventional assay. The line through the points is a linear least squares regression fit to the data. The equation of this line is $y = 0.898 + 0.974x$ with a regression coefficient r of 0.993. (B) Percent difference of lycopene levels between the conventional assay and the reduced volume assay. Percent difference is defined as the lycopene content determined by the reduced volume assay minus that determined by the conventional assay, and that difference divided by the average of the two assay procedures. The equation of the linear regression to the data is $y = -0.284 - 0.012x$ with a regression coefficient r of 0.057.

deteriorate, and it is advisable to keep the tissue chilled and in subdued light while handling. As discussed earlier, the weight of samples for assay must be determined to at least the nearest 0.01 g. For a 0.5 g sample, this represents a weighing uncertainty of ±2% and is the maximum level of experimental error that should be allowed at the weighing step. Because all of the lycopene is extracted into the hexane phase and its weight per volume concentration is determined via absorbance, the volume of hexane delivered to the assay mixture must also be accurate and precise. This can only be accomplished by using a "calibrated to deliver" dispensing system.

CONCLUSIONS

This study describes a rapid and inexpensive way to estimate the lycopene content of certain foods and food products naturally rich in lycopene. We have demonstrated with watermelons that the amount of lycopene in tissue can be determined reliably with a method that employs only 20% of the organic solvents used in conventional spectrophotometric assays. At the same time, the reduced volume extraction method gives statistically identical results with conventional extraction procedures such as those of Sadler *et al.* (1990).

As a measure of what this reduced volume extraction assay can save the researcher in solvent acquisition and disposal costs, we estimate that at current prices each assay costs approximately $0.46 by the conventional procedure and only $0.09 by the reduced volume procedure. This represents not only an 80% cost saving, but an 80% reduction in negative environmental impact as well.

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